

Partial purification and characterization of various β -glucosidases associated with cellular components of corn roots

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β -Glucosidases were isolated from various cellular components from corn root cortical tissue. The subcellular components were separated and the enzymes were partially purified and characterized. The pH-activity profile of the cytosolic enzyme and the unidentified particulate-associated β -glucosidase were identical. Further kinetic analyses of these two enzymes at pH 5.5 and 7.5 suggests that the unidentified particulate-associated β -glucosidase is the bound form of the cytosolic enzyme. The ionically bound cell wall enzyme (in muro) had a pH optimum of 5.5 while the free form (salt solubilized) had a pH optimum of 5.0. The enzyme kinetics of the extracellular enzymes were compared to the intracellular forms with particular reference to bound versus free enzymes. The K_m values and the shape of the kinetic curves of free enzymes were not changed when enzymes were assayed in the bound state. The tightly bound cell wall enzyme had a very broad pH-activity profile and had a K_m value approximately one-half the K_m value of the other β -glucosidases.

Key words: cell wall; bound enzymes; solubilized enzymes; β -glucosidase kinetics; pH optima

Introduction

Although β -glucosidases are located in several compartments in plant cells [1–3], these isozymes have not been separated and characterized in a comparative study. Most studies have focused either on the salt solubilized cell wall-associated β -glucosidase [4–6] or the cytosolic enzyme [3,7]. Little information is available on the kinetic properties of the bound or immobilized enzyme compared with the free or solubilized form and studies of the bound enzyme are needed [8]. Our previous work indicated that at least three different sources of β -glucosidase activity exist in corn root cells.

The cytosol [2], an unidentified particulate fraction [1], and the cell wall [9] all contained β -glucosidase activity. Corn root cell walls have at least two types of β -glucosidase. One is ionically bound and can be removed by salt treatment and the other is tightly bound and cannot be removed with 3 M LiCl. In this report, we have separated and partially characterized the various β -glucosidases to determine if the enzymes are kinetically distinct. Special emphasis was placed on the kinetic properties of bound enzymes compared with their free forms since the binding of enzymes to an immobilized matrix such as the microtrabecular lattice [10] or cell walls [11] may affect enzyme kinetics and hence regulate enzyme activity. Corn root cortical cells are ideal for this study since they have two different particulate enzymes [1,9] that can be analyzed either bound or free.

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Materials and Methods

Plant material

Corn (*Zea mays* L.) seeds (FRB-73)* were surface sterilized, germinated, and harvested as described previously [12]. Root tips were removed and the cortex was physically removed from the stele [12].

Isolation of various β -glucosidases

Isolated cortices (6–10 g fresh wt.) were frozen in liquid nitrogen and ground with a mortar and pestle to generate small clumps of cortical tissue. The ground tissue was suspended in 35 ml of homogenization medium and placed in a Parr nitrogen bomb as described previously [12]. The small capacity Parr bomb was pressurized to 1500 lb/in.² and placed on a Stir Kool Model SK 12 cooling stir plate at 4°C for 15 min. After extrusion from the Parr bomb, the cell walls were trapped on four layers of cheesecloth and washed twice before sonication. After sonication, the cell walls were washed six times with cold deionized-distilled water to remove cytoplasmic contaminants. These walls are highly purified as shown previously [12]. Purified cell walls were extracted with 3 M LiCl for 15 h at 4°C and the extract was used as the free form of the ionically bound cell wall enzyme. The extract was concentrated in an Amicon pressure apparatus with a PM-10 membrane and then dialyzed for 36–48 h at 4°C against deionized-distilled water. The cell walls were exhaustively rinsed with cold deionized-distilled water to remove the salt. The β -glucosidase activity remaining with the cell walls after 3 M LiCl treatment was considered to be the tightly bound enzyme. To study the ionically bound enzyme in muro, the β -glucosidase extract was mixed with cell walls that had been previously treated with 6 M LiCl. The 6 M LiCl treatment (15 h at 4°C) denatured the tightly bound enzyme since no activity could be recovered in the supernatant fraction or the cell walls. The rebinding to these walls was done at 4°C in deionized distilled water and after 30 min,

the cell walls were pelleted (1000 \times g for 2 min) and the supernatant fluid was checked for unbound activity. No activity was found in the supernatant fluid and the cell walls were then washed twice with cold deionized-distilled water before enzyme assays in muro.

The unidentified particle-associated β -glucosidase was isolated from a linear sucrose gradient overlaid with a 1000 \times g pellet from ground cortical tissue and centrifuged at 80 000 \times g for 15 h at 4°C [1]. The unidentified fraction equilibrated at 51% (w/w) sucrose as reported earlier [1]. Unlike cell wall associated enzymes, this particulate form of enzyme activity was solubilized or released below pH 6.0. To keep this enzyme in the bound state, a pH > 7.0 was needed [1]. The cytosolic β -glucosidase activity was isolated and purified from the 120 000 \times g supernatant fraction from ground cortical tissue [2].

Biochemical assays

β -Glucosidase activity and β -galactosidase were with measured *p*-nitrophenyl(PNP)- β -glucose and PNP- β -galactose as substrates. Soluble enzymes were assayed as previously described [2] and cell wall bound enzymes assays were performed as recently reported [12]. Protein estimation was by a modified Lowry procedure [13].

Purification of the cytosolic β -glucosidase activity

A sequential series of ammonium sulfate precipitations were initially used to fractionate the 120 000 \times g supernatant fraction from corn cortical cell homogenates. The fraction precipitating between 45 and 75% of saturation contained the highest specific activity and the highest yield of enzyme activity and was used for further purification.

After dialysis of 4°C for 24 h against 50 mM K-phosphate buffer (pH 7.5), the concentrated sample was applied to a Sephacryl S-200 column. Gel filtration was performed in a cold room with a 2.5 \times 51 cm column and was eluted with 50 mM K-phosphate buffer (pH 7.5) in 0.1 M NaCl; 1.0- or 2.8-ml fractions were collected at a flow rate of 30 ml/h. Fractions containing the peak of β -glucosidase activity were combined and dialyzed overnight against 10 mM K-PO₄(pH 7.5).

*Reference to brand and firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

The β -glucosidase was further purified by isoelectric focusing (IEF) with an LKB 8100-1 column (110 ml in volume, water jacketed) at 4°C. The stabilizing sucrose gradient (5–50%, w/w) was formed with an LKB 8121-1 gradient mixer and contained a final ampholyte concentration of 2% (1.4% pH 4–6 and 0.6% pH 3–10). Voltage was adjusted not to exceed 5 W in power during the first 2 h and was then set at 1600 V and 12.5 W for the rest of the time (22 h total). Two-milliliter fractions were collected from the bottom of the column at a flow rate of 65 ml/h.

Polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed on various sources of β -glucosidase activity using the Phast System by Pharmacia. Homogeneous gels (12.5%) and gradient gels (8–25%) were used and enzymes were located in the gels with the 4-methylumbelliferyl (4- μ) derivatives 4- μ - β -glucose and 4- μ - β -galactose as substrates. Gels were submerged in substrate for 1–5 min at room temperature, rinsed with water, submerged in 1 M Na_2CO_3 for 15 s, rinsed with water and viewed in a light box at 366 nm. The fluorescent activity bands were marked with a razor blade and the gels were stained with Coomassie blue in the Phast System development unit.

Results

Purification of the various β -glucosidases

The 45–75% ammonium sulfate fraction contained the highest specific activity and highest yield of the cytosolic enzyme. After dialysis, the concentrated sample was applied to a Sephacryl S-200 column and a single peak of activity was eluted (Fig. 1). Fractions 41–51 were combined and dialyzed overnight against 10 mM K-PO_4 buffer (pH 7.5) and the sample was further purified by IEF (Fig. 2). During the purification process, both PNP- β -glucose and 4- μ - β -glucose were used as substrates for the β -glucosidase. The purification results verified that both substrates could be used to detect activity and, therefore, validated the use of 4- μ - β -glucose to locate β -glucosidase activity in IEF gels or native polyacrylamide gels. The enrichment and recovery of

β -glucosidase activity during the purification is summarized in Table I. During the purification process, residual β -galactosidase activity was always associated with the β -glucosidase (Figs. 1 and 2). This indicated several possibilities including: (1) there are two separate enzymes with very similar properties; (2) the β -glucosidase is a bifunctional enzyme with two active sites; (3) we were simply observing substrate preference for β -glucose by a single enzyme. Native PAGE indicated 3–4 major protein bands but only one band had β -glucosidase and β -galactosidase activity. To detect β -galactosidase activity, the gel had to be incubated up to 15 min at room temperature.

The unidentified particulate source of β -glucosidase was isolated and combined from eight separate sucrose gradients. The β -glucosidase activity that was found at 51% sucrose was used as the bound form of the unidentified particulate enzyme. To study this enzyme in the free form, the isolated fraction was titrated to pH 6.0 to release the enzyme [1]. Gel filtration and IEF of the free enzyme produced results virtually the same as in Figs. 1 and 2. Native gel electrophoresis confirmed the presence of only one activity band.

The ionically bound cell wall enzyme was salt extracted with 3 M LiCl (57% of the total cell wall associated activity was ionically bound while the rest was tightly bound), dialyzed and used as the free form of the cell wall β -glucosidase. Native PAGE indicated only one band of β -glucosidase activity. This free form was allowed to rebind to cell walls treated with 6 M LiCl and was used as the source of the ionically bound cell wall β -glucosidase. The tightly bound enzyme (after 3 M LiCl treatment) could not be removed from the cell wall, was not further purified, and was studied in muro.

pH optima of various β -glucosidases

Although the pH optima for all of the β -glucosidases were between 5.0 and 5.5, the shape of the pH-dependent curve was broader for cell wall-associated enzymes compared to all soluble isozymes (Fig. 3). The ionically bound cell wall enzyme pH optimum was shifted approximately half a pH unit higher compared with the free (salt-solubilized) enzyme (Fig. 3C). This shift was prob-

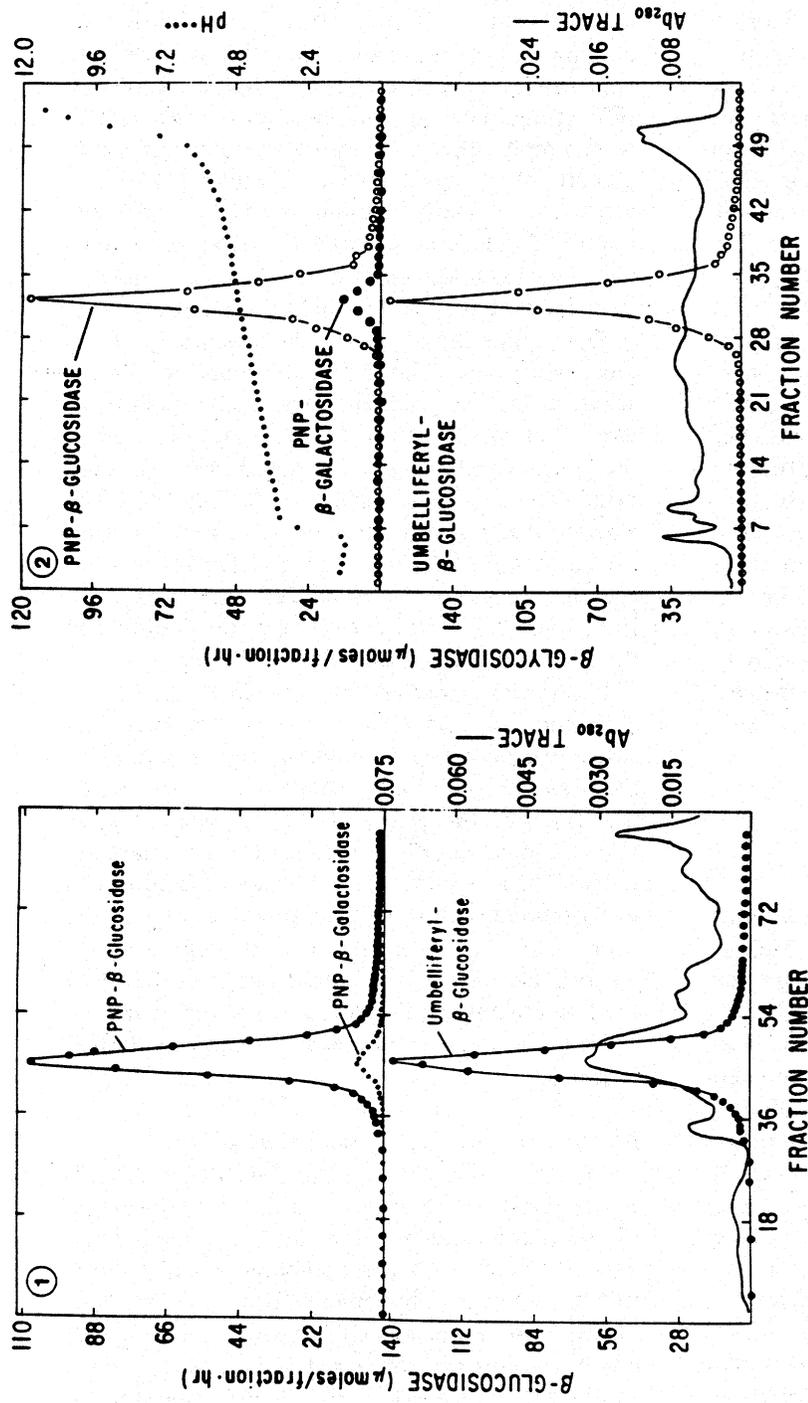


Fig. 1. Gel filtration (Sephacryl 200) of the 40–75% ammonium sulfate fraction precipitated from the 120 000 × g supernatant fraction of a corn root homogenate. β-Glucosidase and β-galactosidase activity were determined with PNP-β-glucose or 4-mu-β-glucose and PNP β-galactose as the substrates.

Fig. 2. Isoelectric focusing of the β-glucosidase activity found in the single peak of Fig. 1.

Table I. Purification of the cytosolic ($120\,000 \times g$ supernatant fraction) β -glucosidase from corn root cortical cells

Fraction	Protein (mg)	TA ^a	Yield (%)	SA ^b	Purification (fold)
120K supernatant ^c	27.80	2081	100	74.9	—
AS(45–75%) ^d	11.42	1330	64	116.5	1.6
Sephacryl S-200 ^e	1.10	612	29	556.4	7.4
IEF ^f	0.21	332	16	1581.0	21.1

^aTotal activity ($\mu\text{mol}/\text{fraction}\cdot\text{h}$).

^bSpecific activity ($\mu\text{mol}/\text{mg protein}\cdot\text{h}$).

^cThe total cellular homogenate that passes through double layered cheesecloth was centrifuged at $120\,000 \times g$ for 30 min at 4°C .

^dPercent of ammonium sulfate saturation (4°C).

^eSee Fig. 1.

^fIsoelectric focusing (see Fig. 2).

ably due to the fact that the pH of the cell wall was more acidic than the pH of the bulk phase [14]. The tightly bound cell wall enzyme (Fig. 3D) had a very broad pH dependent curve and showed that the enzyme was quite active between pH 3.5 and 7.5, with the maximum activity at pH 5.25.

The cytosolic enzyme, the unidentified particulate enzyme, and the solubilized ionically bound cell wall enzyme all had similarly shaped pH profiles (Fig. 3). In this experiment, the unidentified particulate enzyme was free at pH 6.0 and below and was bound at pH-values > 6.8 [10]. No noticeable breaks were observed in this pH dependence curve.

Enzyme kinetics of the various β -glucosidases

The cytosolic β -glucosidase showed typical linear Michaelis-Menten kinetics and the K_m value (Table II) was similar to those reported for the enzyme from other plant tissues [3,7]. All kinetic data were analyzed by the Hanes-Woolf linear transformation of the Michaelis-Menten rate equation.

The unidentified particulate-associated β -glucosidase showed typical Michaelis-Menten kinetics in the free form at pH 5.5. Kinetic analyses were also performed at pH 7.5 to main-

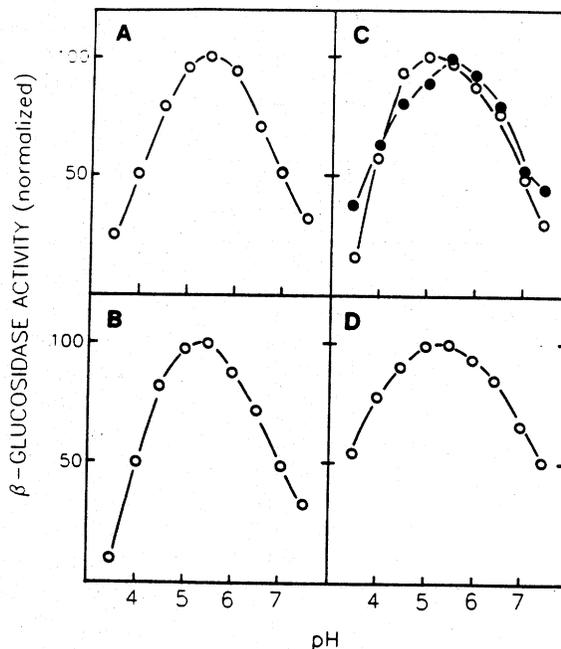


Fig. 3. The pH dependence of activity of β -glucosidases from various subcellular compartments in corn root cortical cells. The cytosolic enzyme, unidentified particulate enzyme, and the cell wall associated enzymes were isolated as described in the text. (A) Unidentified particulate enzyme; (B) cytosolic enzyme; (C) ionically bound cell wall-associated enzyme (\bullet — \bullet). Free or salt solubilized cell wall enzyme (\circ — \circ). (D) Tightly bound cell wall associated enzyme. A 75-mM sodium citrate-citric acid buffer was used and β -glucosidases were assayed with PNP- β -glucose as substrate.

tain the enzyme in the bound form. The K_m value at this pH was similar to the K_m at pH 5.5 (Table II). The cytosolic enzyme also showed an identical small increase in K_m at pH 7.5 compared to pH 5.5. The kinetic analyses, pH optima, and purification during gel filtration and IEF all indicate that the free form of the unidentified particulate enzyme and the cytosolic enzyme are one and the same. The particulate source has not been identified but may represent the microtrabecular lattice [1].

To study the cell wall-associated enzymes, cell wall samples were diluted or concentrated as recently reported [12]. The kinetics of the ionically bound enzyme and its free form were compared at pH 5.5 and the K_m values were identical (Table II). The tightly bound enzyme showed typical

Table II. Michaelis constants determined for various β -glucosidases of corn root cortical cells at different pH values. The K_m values were determined from s/v against s plots and lines were fitted by linear regression analysis with Sigma Plot software. S.E. are in parentheses.

Source of β -glucosidase	pH	K_m (PNP- β -glucose)
Cytosolic	5.5	0.73 (0.04)
	7.5	0.93 (0.02)
Unidentified particulate (free)	5.5	0.75 (0.05)
Unidentified particulate (bound)	7.5	1.02 (0.06)
Cell wall (ionically bound)	5.5	0.88 (0.02)
Cell wall (free)	5.5	0.88 (0.01)
Cell wall (tightly bound)	4.0	0.37 (0.04)
	5.0	0.45 (0.05)
	5.5	0.45 (0.01)
	6.0	0.48 (0.03)
	7.0	0.60 (0.05)
	7.5	0.59 (0.01)

Michaelis-Menten kinetics throughout the pH range tested. All kinetic plots (s/v against s or $1/v$ versus $1/s$) were linear and did not exhibit negative cooperativity between pH 4 and 7.5. A previous study showed that a cell wall-associated β -glucosidase had curvilinear kinetics (negative cooperativity) on Lineweaver-Burk or Eadie-Hofstee plots between pH 5.5 and 7.5 [6].

Discussion

Corn root cells contain several compartments which exhibit β -glucosidase activity. The β -glucosidases were isolated and characterized from these various subcellular sites to determine if the enzymes were kinetically distinct. The major interest was in bound forms of β -glucosidase activity since it has been recently reported that immobilization of enzymes can be viewed as a form of compartmentation or regulation [10]. If the substrate is charged and the enzyme is bound to a similarly charged matrix, non-linear kinetics may be observed because of substrate repulsion. In a recent study (when assayed at low ionic strength), a cell wall bound acid phosphatase had negatively cooperative kinetics while the free enzyme displayed typical Michaelis-Menten kinetics [15].

At high ionic strength, the bound and free enzyme behaved identically as judged from linear kinetic plots [15]. Thus the potential regulation of enzyme activity by ionic strength has been proposed for cell wall associated, membrane bound, and matrix bound enzymes [15–17].

Two reports [4,6] indicated that a cell wall-associated β -glucosidase activity (uncharged substrate so no substrate repulsion) had curvilinear kinetics. In the first case [4], tightly bound β -glucosidase kinetic plots were interpreted as having two distinct cell wall enzymes. Alternatively, their data could be interpreted to indicate one enzyme with negative cooperative kinetics. In the other case [6], curvilinear kinetics were observed for a monomeric β -glucosidase solubilized from cell walls of a soybean cell suspension. Curvilinear kinetics were only observed between pH 5.5 and 7.5; at lower pH-values Michaelis-Menten kinetics were observed. Because pH can influence enzyme kinetics of cell wall associated β -glucosidase and the fact that the pH of the cell wall can change during growth and expansion, enzyme kinetic analyses were performed on the tightly bound wall β -glucosidase at various pH values (Table II). Non-linear or curvilinear plots were not observed at any pH tested and only a small increase in the K_m was noticed with increasing pH. Furthermore, increasing ionic strength at pH 6.0 as in other experiments [18] only slightly increased the tightly bound β -glucosidase activity (data not shown). As the ionic strength of the buffer (0.1 M sodium citrate-citric acid) was increased with the addition of NaCl, the pH of the assay mixture decreased from pH 6.0 (no salt) to pH 5.0 (with 1.2 M NaCl). We interpret this increase in activity with increasing ionic strength to be a result of the drop in pH to where the enzyme is more active (Fig. 3). Ionic strength, in this case, does not appear to regulate the tightly bound cell wall-associated β -glucosidase activity.

Bound enzymes may also exhibit changes in kinetic properties because binding itself may occur at an allosteric site on the enzyme [10]. The bound versus free β -glucosidase in this study did not show any major change in K_m values or shape of the kinetic plots, so the binding of β -glucosidase to a matrix or to the cell wall does not appear to be a form of regulation in corn root cortical cells.

There are, however, corn root cell wall related effects on enzyme activity such as the shift in pH optimum of the ionically bound versus free β -glucosidase (Fig. 3) and the broad pH optimum for the tightly bound β -glucosidase which exhibited considerable activity at either pH 3.5 or 7.5. A recent report with corn roots [19] also indicated that mercury or aluminium inhibition of cell wall-associated acid phosphatase could be alleviated by calcium only when the enzyme was bound. Hence, wall effects may play an important role in regulating cell surface enzyme activity.

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